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Chimeric Antithrombin Peptide

CHARACTERIZATION OF AN ARG-GLY-ASP (RGD)- AND HIRUDIN CARBOXYL TERMINUS-CONTAINING SYNTHETIC PEPTIDE*

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We investigated the properties of an artificial chimeric peptide that contains an Arg-Gly-Asp (RGD)-tripeptide, the versatile cell recognition signal of extracellular matrix protein components, coupled to a carboxyl-terminal fragment of the highly specific α-thrombin inhibitor, hirudin (residues 53–64): WGRGDSANGDFEEIPEEYL (RGD-hirudin$^{53-64}$). Hirudin$^{53-64}$ and RGD-hirudin$^{53-64}$ inhibited the fibrinogen clotting activity of α-thrombin and prolonged the activated partial thromboplastin time of human plasma. In addition, both peptides afforded total protection to thrombin from trypsinolysis. Neither hirudin$^{53-64}$ nor RGD-hirudin$^{53-64}$ dramatically interfered with the thrombin-antithrombin inhibition reaction either in the absence or presence of added heparin. α-Thrombin-induced platelet aggregation was effectively inhibited by hirudin$^{53-64}$ and RGD-hirudin$^{53-64}$. Unlike hirudin$^{53-64}$, RGD-hirudin$^{53-64}$ in solution inhibited integrin-mediated endothelial cell and fibroblast cell attachment to polystyrene wells in the presence of fetal bovine serum. Collectively, our results demonstrate that RGD-hirudin$^{53-64}$ has anticoagulant/antiplatelet aggregation activity attributable to its hirudin sequence and integrin-directed cell attachment activity due to its RGD site. Our results suggest that this chimeric motif may serve as a prototype for a new class of anticoagulants where an integrin-specific sequence "targets" the peptide to a cell (ultimately through the platelet integrin $\alpha_{IIb}\beta_3$) trapped amid a thrombus with ensuing proteinase inhibition.

Hirudin is a highly specific α-thrombin inhibitor isolated from the salivary gland of the European bloodsucking leech *Hirudo medicinalis* (1–3). Recent structure-function studies have shown that both amino- and carboxyl-terminal domains of hirudin bind to thrombin, and the isolated hirudin domains inhibit thrombin through different mechanisms (4–9). The amino-terminal hirudin domain binds to the active site of thrombin, whereas the carboxyl-terminal hirudin fragment binds to the fibrinogen recognition site (adjacent to the active site) (4–9). Only a small portion of the carboxyl terminus of hirudin is required for anticoagulant activity; the minimal peptide length being about 12 amino acid residues (Asn^{53} to Leu^{64}) (4, 5). Hirudin and its fragments have different biochemical properties as potential therapeutic anticoagulants that could favor one over another based on the desired pharmacological characteristics.

Adhesion of blood platelets to vessel wall components and their subsequent activation is a central hemostatic event. An essential component of platelet adhesion and aggregation is the cell surface receptor $\alpha_{IIb}\beta_3$ (also known as glycoprotein IIb-IIIa), which is a member of the integrin family (10–14). Platelet $\alpha_{IIb}\beta_3$ is a receptor for four adhesive proteins: fibrinogen, fibronectin, vitronectin, and von Willebrand factor (10, 11, 13). $\alpha_{IIb}\beta_3$ specifically recognizes a conserved tripeptide Arg-Gly-Asp (RGD) sequence found in all four proteins and the carboxyl terminus of the γ chain of fibrinogen (HHLGGAKQAGDV) (10, 11, 13, 15). Additionally, there are many other integrins found in numerous cell types that specifically mediate both cell adhesion with substrates derived from extracellular matrix and body fluids and cell-cell interactions (10, 16).

There are examples of hybrid molecules either that combine two functions or that acquire a new function. A bifunctional thrombin inhibitor has been prepared by linking (D-Phe)-Pro-Arg-Pro- and hirudin carboxyl-terminal fragments (17, 18). RGD- and HHLGGAKQAGDV-containing sequences either coupled to or genetically engineered into a carrier protein have integrin-specific cell binding activity similar to the parent adhesive protein (15, 19–21). We hypothesized that an artificial chimeric peptide could be constructed that incorporated antithrombin activity and integrin-directed cell attachment activity. This chimera motif is based on the finding that platelet phospholipid microparticles produced following platelet activation contain the components for assembly of the prothrombinase complex and functional $\alpha_{IIb}\beta_3$ receptors (22). Therefore, coupling these sequence types into a chimera might provide a "targeted" antithrombin agent to specifically bind cells at a thrombus for inhibition of thrombin.

In the present investigation, we synthesized a chimeric peptide by adding the RGD tripeptide, a minimal cell adhesion sequence from fibronectin and other adhesive proteins, to a segment of the carboxyl terminus of hirudin (termed *chimeric antithrombin peptide*). We report here that the chimeric antithrombin peptide has both antithrombin and cell adhesion activities comparable to its individual constituents.

EXPERIMENTAL PROCEDURES

Materials—All *N*-(9-Fluorenyl)methoxycarbonyl-amino acid derivatives and reagents were obtained from Milligen/Cambridge Research Biochemicals. Human α-thrombin and antithrombin were purified as described (23, 24). Heparin was provided by Diosynth; L1-

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tosylamido-2-phenylethyl chloromethyl ketone-treated trypsin was from Cooper Biochemicals. Bovine fibrinogen was from Miles Laboratories; Chromozyme TH (tosyl-Gly-Pro-Arg-*p*-nitroanilide) was obtained from Boehringer Mannheim.

Peptide Synthesis—Peptides were assembled using a Milligen Pep-synthesizer as described previously (25). Purity of the peptides was analyzed by reverse-phase HPLC[1] (24, 26), and if necessary, peptides were further purified by HPLC on a preparative Vydac C_{18} column. All peptides were analyzed either by amino acid analysis or by primary structural analysis on an Applied Biosystems 475A Protein Sequencer (Protein Chemistry Laboratory, Department of Chemistry of this institution). An excellent correlation between expected and actual values/sequences was found for all peptides. Sequences of synthetic hirudin$^{53-64}$ and RGD-hirudin$^{53-64}$ peptides are shown below (picomoles of amino acid yield/cycle are shown in parentheses).

Hirudin$^{53-64}$: H-Asn(409)-Gly(628)-Asp(809)-Phe(645)-Glu(410)-Glu(311)-Ile(350)-Pro(257)-Glu(168)-Glu(140)-Tyr(119)-Leu(73)-Ala(29)-OH

RGD-hirudin$^{53-64}$: H-Trp(1150)-Gly(1166)-Arg(965)-Gly(715)-Asp(599)-Ser(479)-Ala(734)-Asn(308)-Gly(339)-Asp(435)-Phe(430)-Glu(263)-Glu(250)-Ile(237)-Pro(227)-Glu(154)-Glu(138)-Tyr(67)-Leu(6.4)-OH.

All other peptides and their sequences using the one-letter abbreviation (shown in parentheses) were as follows: RGD-peptide (GRGDSAY); RGE-peptide (YARGESA); RGE-hirudin$^{53-64}$ (WGRGESANGDFEEIPEEYL); and HC$^{39-66}$ (DFHKENTVTND-WIPEGEEDDDYLDLEKIY) (25).

Anticoagulant and Antithrombin Assays—All experiments were performed in a buffer that contained 20 mM HEPES, 150 mM NaCl, 0.1% (w/v) polyethylene glycol (M_r = 8000) at pH 7.4. Fibrinogen clotting activity of human α-thrombin was measured in bovine serum albumin-coated microtiter plates by incubating 50 μl of thrombin (10 nM stock) with 50 μl of a synthetic peptide (8–20 μM stock). After 1 min, 100 μl of fibrinogen (5 mg/ml stock) was added, briefly agitated, and the absorbance at 405 nm was measured every 5 s for 2 min in a V_{max} kinetic microplate reader (Molecular Devices). These experiments were performed in triplicate from three to five times. aPTT of pooled human plasma using Thromboscreen Kontact reagent (Pacific Hemostasis) was determined in the presence of synthetic peptides with a Fibrometer as described (27). Experiments were performed three times and the results averaged.

Antithrombin inhibition assay of thrombin in the presence of a 200-fold molar excess of either hirudin$^{53-64}$ or RGD-hirudin$^{53-64}$ to thrombin was performed as described previously (27). Thrombin inhibition by antithrombin-heparin in the presence of synthetic peptide was performed by incubating 1 nM thrombin with 100 nM hirudin$^{53-64}$ for 1 min, followed by 10 nM human antithrombin in the presence of 0.05 to 500 μg/ml heparin. After 20 s, Chromozyme TH with polybrene (to neutralize the added heparin) was added, substrate hydrolysis was stopped after 60 min by the addition of glacial acetic acid, and the absorbance at 405 nm was determined. Inhibition rate constants were calculated as detailed previously (27). These experiments were performed three times.

Proteolysis of α-Thrombin by Trypsin—Trypsinolysis of α-thrombin was performed with 2.75 μM thrombin (100 μg in 100 μl) in the presence of 125 μM hirudin$^{53-64}$ or RGD-hirudin$^{53-64}$ in HEPES-buffered saline, pH 7.4. After a 5-min incubation at room temperature, the reaction was initiated by the addition of 2 μg of L1-tosylamido-2-phenylethyl chloromethyl ketone-treated trypsin and stopped after 120 min by the addition of 1 mM phenylmethylsulfonyl fluoride. The extent of proteolysis was assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis in 15% slab gels without chemical reduction of samples. Silver nitrate was used to stain the polypeptides. This experiment was performed three times.

Platelet Aggregation Assay—Platelet aggregation assays were performed using human platelet-rich plasma (diluted to 300,000 platelets/μl) by drawing blood (9 parts) into 3.8% (w/v) sodium citrate (1 part) from a volunteer who had not had any aspirin or related products for at least 14 days. Platelet aggregation was performed by adding 40 μl of a synthetic peptide solution to 450 μl of platelet-rich plasma at 37 °C. After a 2-min incubation, 10 μl of α-thrombin (0.4 μM NIH unit/ml final concentration) was added and the light transmittance (Bio/Data PAP-4 Aggregometer) was recorded. These experiments were performed in triplicate four times with four different healthy volunteers and the results averaged.

Cells and Cell Attachment Assays—Human dermal fibroblasts (supplied by Dr. R. A. Briggaman, Department of Dermatology of this institution) were grown in DMEM (GIBCO) supplemented with 10% FBS, 100 units/ml penicillin and 100 μg/ml streptomycin. The human endothelial cell line (EA.hy 926; supplied by Dr. C-J. S Edgell of this institution) was grown as described previously (28). Cell adhesion activity of the synthetic peptides was determined as described (29). Briefly, ~1×10^5 cells/ml of trypsinized cells were mixed with DMEM containing 10% FBS and 0.5, 0.5, 1.0, 1.5, and 1.5 mg/ml of RGD, RGE, hirudin$^{53-64}$, RGD-hirudin$^{53-64}$, or RGE-hirudin$^{53-64}$ peptides, respectively (these concentrations provided approximately equal molar amounts of RGD/E). Cells that attached to the microtiter plate wells after incubation for 60 min at 37 °C and 5% CO_2 were quantified by staining with Crystal Violet, solubilizing the stained cells with ethylene glycol monomethyl ether, and the absorbance at 600 nm was compared with standard curves of serially diluted cells (30). These experiments were performed from three to six times.

RESULTS

Anticoagulant and Antithrombin Activities—Coupling the cell adhesive RGD sequence (and the inactive RGE conformer) to hirudin$^{53-64}$ did not affect the ability of hirudin$^{53-64}$ to inhibit fibrinogen hydrolysis by thrombin (Fig. 1, *top*). The concentration required for 50% inhibition (IC_{50}) for hirudin$^{53-64}$, RGD-hirudin$^{53-64}$, and RGE-hirudin$^{53-64}$ was ~0.6 μM. There was also a dose-dependent increase in the aPTT of normal pooled human plasma (average clotting time of 40 + 1.3 s for 100% plasma) for hirudin$^{53-64}$, RGD-hirudin$^{53-64}$,

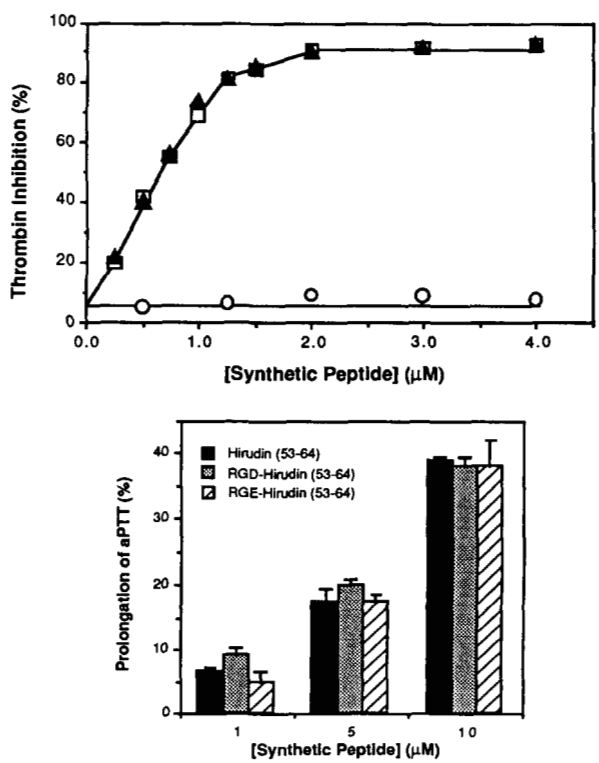

FIG. 1. **Anticoagulant and antithrombotic activities of synthetic peptides.** *Top*, fibrinogen clotting activity of human α-thrombin was measured as described under "Experimental Procedures": hirudin$^{53-64}$ (□) and RGD-hirudin$^{53-64}$ (▲). RGE-hirudin$^{53-64}$ had essentially the same activity as shown here for hirudin$^{53-64}$ and RGD-hirudin$^{53-64}$ (data not included). A control peptide, HC$^{39-66}$ (○), did not inhibit fibrinogen clotting activity. *Bottom*, aPTT assays were performed using normal human pooled plasma as detailed under "Experimental Procedures." The control HC$^{39-66}$ peptide did not prolong the aPTT (tested at 10 μM).

[1] The abbreviations used are: HPLC, high performance liquid chromatography; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; aPTT, activated partial thromboplastin time; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum.

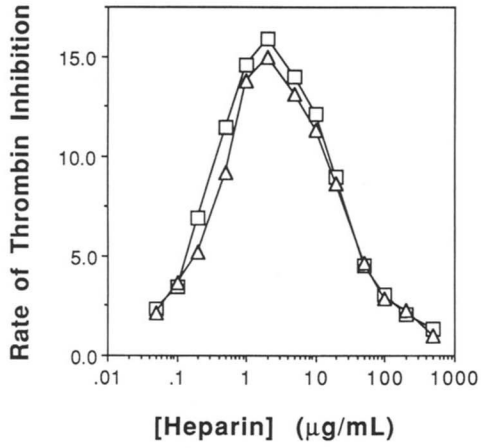

FIG. 2. **Effect of hirudin$^{53-64}$ on thrombin inhibition by antithrombin-heparin.** Thrombin inhibition by antithrombin in the presence of various amounts of heparin was performed as detailed under "Experimental Procedures" either in the absence (□) or in the presence (△) of hirudin$^{53-64}$. Thrombin inhibition was determined as the second-order rate constant of inhibition ($\times 10^{-6}$ M^{-1} s^{-1}).

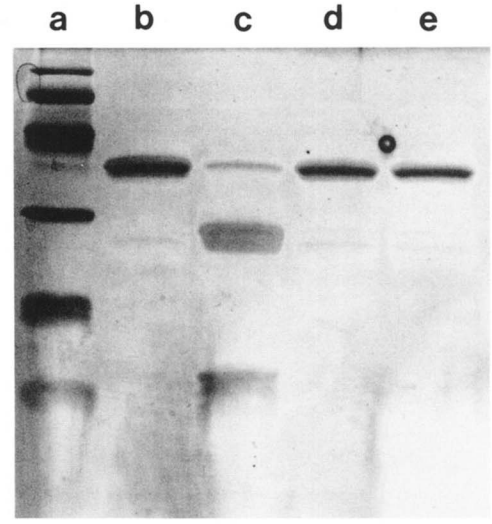

FIG. 3. **Effect of hirudin$^{53-64}$ and RGD-hirudin$^{53-64}$ on trypsinolysis of α-thrombin.** Trypsinolysis of α-thrombin was performed in the absence and presence of hirudin$^{53-64}$ and RGD-hirudin$^{53-64}$ as described under "Experimental Procedures" with assessment of proteolysis by gel electrophoresis. Lanes: *a*, Bio-Rad low molecular weight standards; *b*, thrombin alone; *c*, thrombin plus trypsin; *d*, thrombin plus trypsin and hirudin$^{53-64}$; and *e*, thrombin plus trypsin and RGD-hirudin$^{53-64}$.

and RGE-hirudin$^{53-64}$ (Fig. 1, *bottom*).

We examined thrombin inhibition by the plasma serpin antithrombin in the presence of hirudin$^{53-64}$ and RGD-hirudin$^{53-64}$. Neither hirudin$^{53-64}$ nor RGD-hirudin$^{53-64}$ interfered with the thrombin-antithrombin inhibition reaction (in the absence of added heparin) as shown by second-order rate constants of 1.37, 1.22, and 1.34 ($\times 10^5$ M^{-1} min^{-1}) in the absence of peptide, and in the presence of a 200-fold molar excess of hirudin$^{53-64}$ or RGD-hirudin$^{53-64}$ to thrombin, respectively.

We also determined the effect of hirudin$^{53-64}$ on thrombin inhibition by antithrombin-heparin. At a 100-fold molar excess of hirudin$^{53-64}$ to thrombin, there was essentially no difference in the rate of thrombin inhibition by antithrombin in the presence of various amounts of heparin (Fig. 2).

Trypsin hydrolyzes α-thrombin at unique sites in the B-chain to form β_T- and γ_T-thrombin derivatives. We assessed the effect of hirudin$^{53-64}$ and RGD-hirudin$^{53-64}$ on trypsinolysis of α-thrombin. Both hirudin$^{53-64}$ and RGD-hirudin$^{53-64}$ afforded essentially total protection to α-thrombin during incubation with trypsin (Fig. 3). Control experiments verified that the hirudin$^{53-64}$-containing peptides had no inhibitory effect on trypsin.

We examined the peptides for dose-dependent inhibition of platelet aggregation in α-thrombin-stimulated human platelets. Platelet aggregation induced by α-thrombin was inhibited most effectively by hirudin$^{53-64}$, RGD-hirudin$^{53-64}$, and RGE-hirudin$^{53-64}$ (IC_{50} of 7 μM for each peptide), but less effectively by the RGD-peptide (IC_{50} ~100 μM), and with no effect by the RGE-peptide (tested to 300 μM).[2] Complete inhibition of α-thrombin-induced platelet aggregation was observed with 15 μM hirudin$^{53-64}$, RGD-hirudin$^{53-64}$, and RGE-hirudin$^{53-64}$.

These data indicate that the fibrinogen clotting and platelet aggregation activities of α-thrombin (in a purified or plasma-based assay system) are inhibited to essentially the same extent by hirudin$^{53-64}$ and RGD/E-hirudin$^{53-64}$ and that addition of the RGD/E sequence to hirudin$^{53-64}$ is not detrimental to its anticoagulant and antithrombin activities. The data also suggest that hirudin$^{53-64}$ and RGD-hirudin$^{53-64}$ bind to the same site on thrombin since neither influences inhibition by the plasma serpin antithrombin and both protect thrombin during trypsinolysis.

TABLE I
Inhibition of cell adhesion by synthetic peptides

Peptide	Cell adhesion (absorbance 600 nm)[a]	
	Endothelial cells	Fibroblasts
	%	
RGD	53 (0.121 ± 0.007)	34 (0.097 ± 0.003)
RGD-hirudin$^{53-64}$	45 (0.103 ± 0.007)	37 (0.103 ± 0.006)
RGE	96 (0.219 ± 0.056)	79 (0.223 ± 0.017)
Hirudin$^{53-64}$	91 (0.207 ± 0.020)	81 (0.229 ± 0.024)
RGE-hirudin$^{53-64}$	92 (0.209 ± 0.073)	80 (0.225 ± 0.010)

[a] Cell adhesion was measured by Crystal Violet staining of cells as described under "Experimental Procedures." The data are from a representative experiment and they are consistent with the trend in all experiments evaluating these peptides to inhibit cell adhesion in the presence of FBS. The 100% cell adhesion value was arbitrarily assigned to the lowest dilution of cells (~1×10^4 cells) in the standard curve which gave absorbances at 600 nm of 0.228 and 0.262 for endothelial cells and fibroblasts, respectively.

Cell Adhesion Activity—We compared each synthetic peptide in solution for its ability to inhibit fibroblast and endothelial cell attachment in the presence of FBS. Cell surface integrins will bind to RGD-containing adhesive proteins present in serum as FBS coats the microtiter plate surface. We found that RGD-hirudin$^{53-64}$ and the RGD-peptide were quite effective at preventing cell attachment, whereas hirudin$^{53-64}$, the RGE-peptide, and RGE-hirudin$^{53-64}$ did not interfere with cell adhesion (Table I). Microscopic inspection of fibroblasts and endothelial cells verified that hirudin$^{53-64}$, the RGE-peptide, and RGE-hirudin$^{53-64}$ had no noticeable effect on cell attachment. However, RGD-hirudin$^{53-64}$ and the RGD-peptide did affect adhesion in that the cells were rounded and not attached to the surface (shown for fibroblasts in Fig. 4). These data demonstrate that RGD-hirudin$^{53-64}$ functions like the

[2] RGD-containing peptides prevent platelet aggregation by inhibiting fibrinogen binding to activated platelets (10–14). Thus, in addition to testing the hirudin site of RGD-hirudin$^{53-64}$ to block thrombin-mediated platelet aggregation, we also compared the synthetic peptides for dose-dependent inhibition in ADP-stimulated platelets. There was a similar concentration dependence for the RGD-peptide and RGD-hirudin$^{53-64}$ to inhibit ADP-stimulated platelet aggregation (J. L. Woods and F. C. Church, unpublished observation).

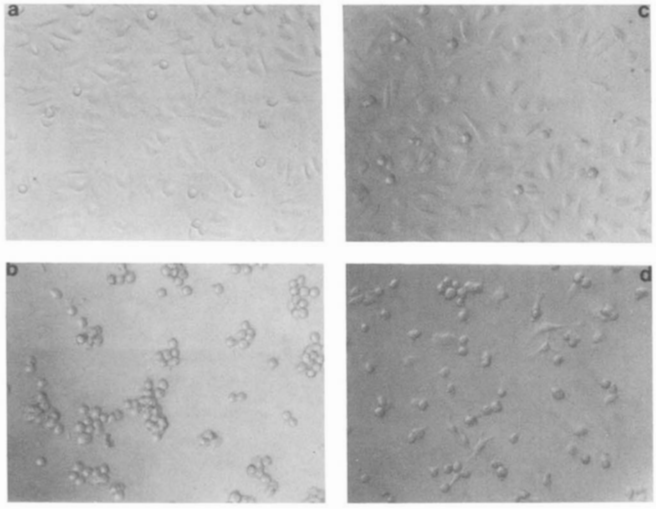

FIG. 4. **Effect of synthetic peptides on fibroblast adhesion.** Inhibition of fibroblast cell adhesion in the presence of DMEM/FBS with and without synthetic peptides was performed as described under "Experimental Procedures." *a*, DMEM/FBS alone; *b*, DMEM/FBS and the RGD-peptide; *c*, DMEM/FBS and hirudin$^{53-64}$; and *d*, DMEM/FBS and RGD-hirudin$^{53-64}$.

RGD-peptide in inhibition of cell adhesion, but that the hirudin$^{53-64}$ sequence alone does not affect cell adhesion.

We investigated whether RGD-hirudin$^{53-64}$ could act as a "bridge" between RGD-specific cell receptors and thrombin (as a replacement for the adhesive proteins present in FBS). We prepared thrombin complexes with RGD-hirudin$^{53-64}$ and hirudin$^{53-64}$ and adsorbed the thrombin-peptide complexes to polystyrene; next, fibroblasts were added in the absence of FBS, and the number of fibroblasts attached and spread were determined. Interestingly, thrombin alone or in complex with the peptides promoted the adhesion and spreading of fibroblasts to ~70% of the adhesion observed with fibronectin (data not included). It should be noted that the B-chain of thrombin has an RGD sequence which apparently acts as an adherent substrate (31). Thus, we were not able to demonstrate the coordinating activities of cell adhesion and thrombin inhibition with our initial chimeras. This could be due to not only the "active" RGD adhesion sequence in thrombin for fibroblasts (31) or the actual amount of an RGD-peptide necessary to support cell adhesion (10) but also possibly to the absence of an adequate "spacer" separating the two active sites in this chimera.

DISCUSSION

This study was undertaken to characterize a chimera combining RGD and hirudin sequences. Our results with RGD-hirudin$^{53-64}$ are in accord with previous observations using hirudin carboxyl-terminal fragments in anticoagulant, antithrombin, and platelet aggregation inhibition assays (4–9, 32–35). Our data and those of others indicate that hirudin carboxyl-terminal fragments bind to the fibrinogen recognition site (anion exosite domain) of thrombin which effectively blocks both fibrinogen clotting and thrombin-stimulated platelet aggregation activities. These hirudin fragments also do not affect thrombin inhibition by the serpin antithrombin with or without heparin. Thus, hirudin fragments that are targeted to the anion exosite of thrombin, not the active site, may work independently of antithrombin to regulate thrombin. Future chimeric antithrombin peptide designs for the hirudin site will include variations in the sequence of the carboxyl-terminal fragment (36) and specific chemical modification of Tyr^{63} (either by nitration (37), iodination (37), or sulfation (5)) in an effort to increase its overall antithrombin/anticoagulant potency.

Our results demonstrate that RGD-hirudin$^{53-64}$ has the same cell-binding activity as the RGD-peptide alone; thus, RGD in the RGD-hirudin$^{53-64}$ chimera must assume an active conformation. Many proteins have been identified that contain the RGD tripeptide sequence, but the presence of an RGD sequence does not necessarily confer cell adhesion activity (10). There is sufficient evidence to suggest that both RGD conformation and environment contribute to integrin-directed cell recognition (10, 13, 20, 29, 38–41). This recognition specificity (and affinity) for RGD-containing peptides/proteins implies that a unique sequence can be "engineered" to preferentially interact with a particular integrin (for instance, by stereochemical isomerization, cyclization, or a unique next-neighbor sequence). Indeed, RGD peptide-albumin conjugates have been shown to recognize specific integrins (19, 21).

$\alpha_{IIb}\beta_3$ is the dominant fibrinogen receptor in platelets (10–14). Cross-linking studies have shown that RGD binds predominantly to the β_3 subunit, whereas HHLGGAKQAGDV binds to the α_{IIb} subunit. There are other fibrinogen receptors including the $\alpha_v\beta_3$ integrin found primarily on endothelial cells (40) and $\alpha_M\beta_2$ on leukocytes (42). Comparison of the binding specificity of $\alpha_{IIb}\beta_3$ and $\alpha_v\beta_3$ for fibrinogen shows that $\alpha_{IIb}\beta_3$ preferentially recognizes both an RGD-peptide modeled after Aα^{93-95} RGD (but not A$\alpha^{572-574}$ RGD) and γ-chain$^{400-411}$ HHLGGAKQAGDV sequences, whereas $\alpha_v\beta_3$ exclusively interacts with an RGD-peptide modeled after the A$\alpha^{572-574}$ RGD sequence (40). There are many other integrins that interact with different protein sites than those just described, for instance, leukocyte $\alpha_M\beta_2$ binds a novel fibrinogen site (neither RGD nor the carboxyl terminus of the γ-chain) (42) and $\alpha_4\beta_1$ in a melanoma cell line recognizes a fibronectin sequence consisting of *X*-Asp-*Y* (43). Therefore, it would appear that appropriate peptide sequences can be designed to specifically target the chimera to platelet $\alpha_{IIb}\beta_3$ and not other integrins capable of binding fibrinogen or other proteins.

Previous studies have shown that RGD-and HHLGGAKQAGDV-containing peptides prevent fibrinogen binding to platelets and platelet aggregation and alter the conformation of purified platelet $\alpha_{IIb}\beta_3$ (10–15).[2] These peptide sequences have been implicated as potential candidates for therapeutic antiplatelet agents (13, 14). Furthermore, a family of RGD-containing proteins from a variety of snake venoms and leeches has recently been described as potent antiplatelet compounds (44, 45). Future chimeric antithrombin peptide designs for the integrin-directed site (platelet $\alpha_{IIb}\beta_3$) will incorporate unique/specific RGD sequences (such as that in the fibrinogen Aα^{93-95} chain or that found in the snake venom RGD-protein family) and non-RGD sequences (such as that in the fibrinogen γ-chain$^{400-411}$ sequence).

The possibility for an achievable targeted chimeric antithrombin peptide is strengthened by Bode *et al.* (46), Sandberg *et al.* (47), and more recently by the work of Sims *et al.* (22) in their detection of both functional prothrombinase complex components and $\alpha_{IIb}\beta_3$ incorporated into platelet plasma membrane microparticles. We ultimately envision a chimeric antithrombin peptide combining $\alpha_{IIb}\beta_3$-specific and thrombin anion exosite-directed active sites. This peptide would be capable of interacting with stimulated platelets trapped within a thrombus and not only blocking platelet-fibrinogen (or other RGD-containing proteins) interactions but also halting thrombin-mediated fibrinogen clotting and platelet aggregation activities. Finally, the partnership of distinct/different

target sites in these chimeras might support cooperative multifunctional activities.

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